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SOME EFFECTS OF EYESTALK REMOVAL ON THE Y-ORGANS OF *CANCER IRRORATUS* SAY

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Brachyuran crustaceans possess a pair of endocrine glands called Y-organs which are thought to be homologous to the molting glands of insects (Gabe, 1953). Bilateral removal of these Y-organs prevents molting in *Carcinus maenas* (Echalier, 1954) and *Sesarma reticulatum* (Passano and Jyssum, 1963); and reimplantation leads to the resumption of the normal molting process (Echalier, 1955). Bilateral eyestalk removal in the fiddler crab, *Uca pugilator* (Abramowitz and Abramowitz, 1940), has been shown to lead to precocious molting when it is performed during the intermolt period of the molt cycle, but not when performed during the premolt period in *Sesarma reticulatum* (Passano and Jyssum, 1963). It appears that the decapod crustacean eyestalk neurosecretory complex secretes a hormone that inhibits the Y-organ from producing a molting hormone (Passano, 1960; Charniaux-Cotton and Kleinholz, 1964). Eyestalk removal or destalking appears to result in the hypertrophy of Y-organ cells as evidenced in *Hemigrapsus nudus* (Matsumoto, 1962) and *Libinia emarginata* (Hinsch, 1973). Yet an important consideration is whether or not this hypertrophy is indicative of cellular activity. One method of monitoring Y-organ activity is quantification of RNA synthesis as measured by the uptake of a radioactive precursor, ^{3}H-uridine (Comings, 1966; Brasiello, 1968; Gorell and Gilbert, 1969; Foulks and Hoffman, 1974). The following experiment was designed to test whether there is any statistically significant difference in the incorporation rate of ^{3}H-uridine into the Y-organ cells of a crab at various time periods following bilateral eyestalk removal.

Materials and Methods

Female specimens of the East Coast rock crab, *Cancer irroratus* Say, were obtained from Sheepscot Specimen Company, West Southport, Maine. The animals were maintained in a 25 gallon Instant Ocean Culture System at 10° to 11° C and 35 to 37 ppt salinity. The crabs were regularly fed fish fillets prior to the beginning of the experiments. The destalking experiments were undertaken from October 7th through December 7th, 1973; and the 75 animals, all non-ovigerous, that were used in these experiments ranged in weight from 40 to 148 g.

Both eystalks were removed by severing them at their bases with a fine pair of scissors. The wounds were not cauterized and the crabs were allowed to rest 12 hours to recover from the trauma of the surgery. A control group of animals (Group 1) was not destalked. There were five groups of bilaterally destalked animals, Groups 2 through 6. These animals were destalked 1.5, 2.5, 4.5, 7.5 and 10.5 days, respectively, prior to being injected with the labeled uridine. Both

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control and destalked crabs were injected with aqueous solution of ^{3}H-uridine using a one ml tuberculin syringe into the arthrodial membrane at the base of the 4th or 5th left walking leg. The given dose was 0.25 microcuries/g weight animal. The ^{3}H-uridine (NET 174, New England Nuclear, Boston, Massachusetts) had specific activities ranging from 26.1 to 27.8 Ci./mM. The wound was cauterized immediately after injection. All animals were sacrificed 24 hours post injection. Thus the time interval after destalking in which uridine incorporation was measured is one day later than the injection time, *i.e.*, 2.5, 3.5, 5.5, 8.5 and 11.5 days postdestalking. Using the amount of deposition of new cuticle at the time of sacrifice (Passano, 1960), all the control and experimental animals were found to be in premolt.

A glutaraldehyde fixation was used for epoxy sections. Primary fixation in glutaraldehyde and postfixation in osmium are outlined in Hoffman (1969). The tissue was embedded in epoxy resins according to the method of Luft (1961). Thick sections approximately 0.5 to 1.5 microns were made for autoradiographs and for microscopic examination. In the latter case, thick sections were stained with Richardson's stain (Richardson, Jarett and Finke, 1960).

Autoradiographs were prepared by dipping unstained slides into a 50% aqueous solution of Kodak NTB-3 liquid emulsion, and exposing them for 4 weeks at 5° C. They were developed and fixed according to the method outlined by Bogoroch (1972). The autoradiographs were examined using phase contrast microscopy with an oil immersion lens and an ocular with a calibrated grid. The grid consisted of 100 squares each measuring 10 microns on a side at 1000 X. Counts of reduced silver grains were undertaken over five randomly selected areas of each Y-organ. In each of these areas, silver grains were counted in five randomly selected grid squares, giving a total of 500 square microns of tissue area counted. The mean silver grain count for the five different areas of each gland was then determined. Background counting was done in the same manner over areas of epoxy resin without tissue. Background counts were subtracted from the counts made over the tissue. Cell boundaries could not be easily distinguished for direct measurements of the cells. Relative cell sizes in the Y-organs were determined by counting the number of nuclei per 500 square microns of tissue using the same counting technique as that used for counting the reduced silver grains.

Statistical analysis was performed on the differences between the means of the various experimental groups. Wherever significant F values were found using an analysis of variance, significance was tested between groups using multiple t-tests. Since the experimental groups contained unequal numbers of glands further testing was done using the Scheffé test (Scheffé, 1959).

Six Y-organs taken from animals that had been destalked for 2.5 days and then injected with an equivalent dose of ^{3}H-uridine were incubated in RNAase with Millonig's phosphate buffer at 37° C for 1 hour in order to test whether the label was being incorporated into RNA. The mean silver grain counts for these Y-organ sections (4.00 ± 1.43 S.E.) were statistically below the average mean count for the group destalked for 2.5 days (12.14 ± 1.04 S.E.) at $P < 0.05$. Therefore, the concentrated incorporation of ^{3}H-uridine into Y-organ cells was RNAase sensitive and was considered evidence of RNA synthesis.

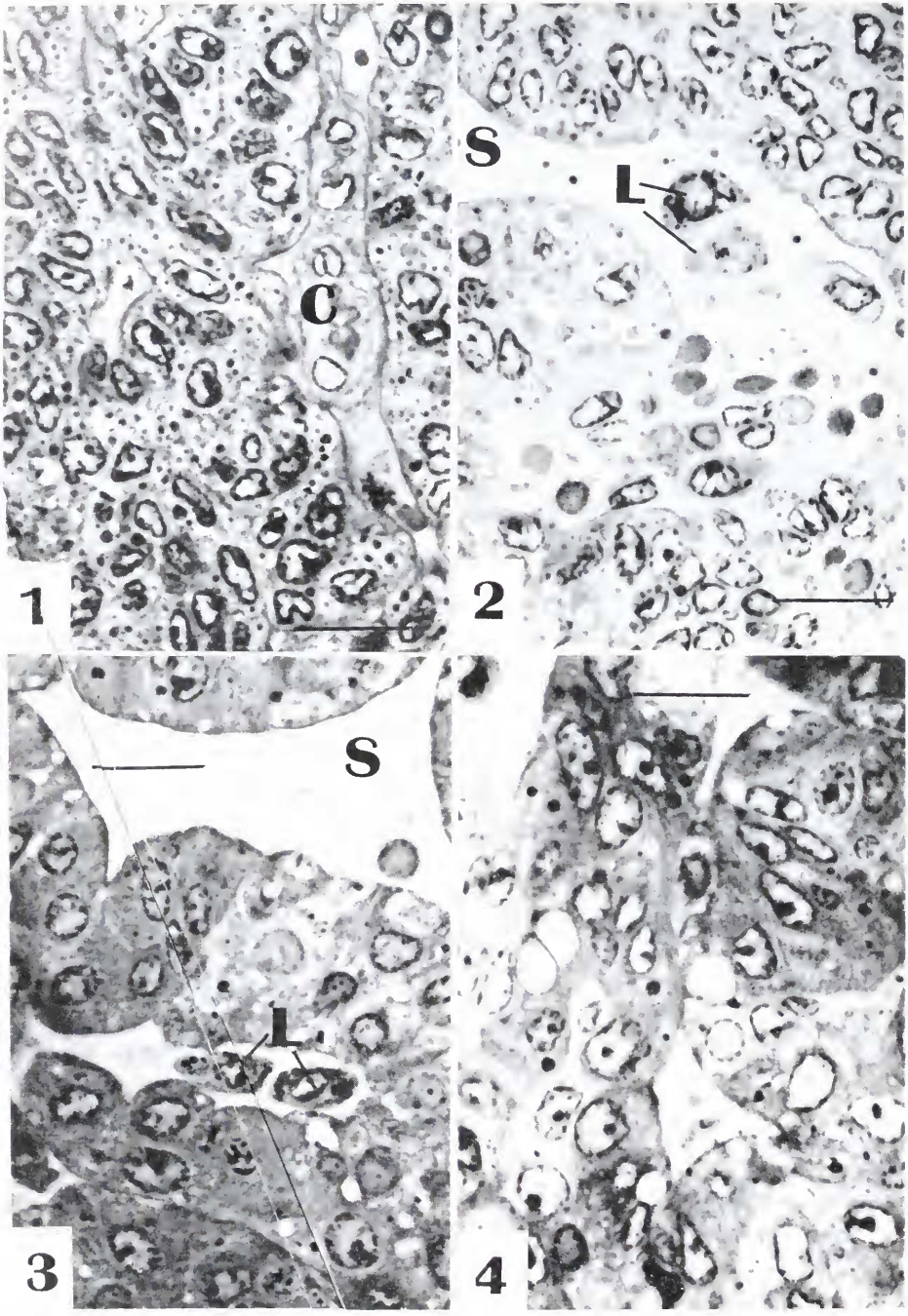

FIGURE 1. Thick epoxy section (1 μ) through the Y-organ of a control (non-destalked) female specimen of *Cancer irroratus*. Note the numerous osmophilic granules within the

Results

The Y-organs of *Cancer irroratus* are found in the same anatomical location as the Y-organs of *Carcinus maenas* as described by Echalier (1959), and they conform to the description of the classical Y-organ of Gabe (1953). They are paired structures, approximately 1 to 2 mm in diameter, light milky yellow in color, that are located in the cephalothorax anterior to the branchial chamber just posterior and immediately lateral to the eyestalks. The glands are in intimate contact with connective and lymphogenous tissue and directly applied to the hypodermis of the ventral carapace at the intersection of two skeletal ridges. These ridges form the insertion of a small accessory mandibular muscle, the "petit muscle lateral" of Echalier (1959) under which lies the Y-organ.

The histology of the Y-organ

The Y-organ consists of anastomosing cords of epithelial cells separated by numerous interconnected hemocoelic sinuses. Numerous fine capillaries are also evident within these sinuses. The cells have irregularly shaped nuclei approximately 8–10 microns in diameter with peripheral chromatin granules and peripheral or centrally located nucleoli. Mitotic activity was not evident in any of the cells. The cytoplasm contains numerous osmophilic granules that can be quite variable in size, some exceeding one micron in diameter (Fig. 1). In glutaraldehyde-fixed tissue that had not been postfixed with osmium tetroxide, these granules appear yellow in color under phase contrast microscopy. The yellowish color of the gland may be due to these granules since no pigment cells are evident in or on the surface of the gland. The cytoplasm of the cells can be quite variable, ranging from very homogeneously staining with Richardson's stain to highly vacuolated. Cellular limits are not readily evident with light microscopy, but the cells appear to range in size from 15 to 20 microns.

The peripheral areas of the gland show evidence of cellular degeneration (Fig. 2). The nuclei of these degenerating cells are ovoid in shape, and many appear pycnotic staining intensely with Richardson's stain. The cytoplasm of these cells is highly vacuolated containing in addition numerous osmophilic granules. In a few of the glands, peripheral cords of cells appear to be rupturing. This is especially evident in regions of cellular degeneration.

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vacuolated cytoplasm of the cells. Also note the small capillaries (C) within the blood sinuses between the cords of cells; Richardson's stain. The slash mark represents 25 microns.

FIGURE 2. Thick epoxy section (½ μ) through the Y-organ of a control (non-destalked) female specimen of *Cancer irroratus*. Note the area of degeneration with pycnotic nuclei at the center. Also the lymphocytes (L) can be seen within the blood sinus (S); Richardson's stain. The slash mark represents 25 microns.

FIGURE 3. Thick epoxy section (1 μ) through the Y-organ of a 3.5 day destalked female specimen of *Cancer irroratus*. Note the hypertrophied cells and the region of cytoplasmic vacuolization. The granular lymphocytes (L) are within a blood sinus (S); Richardson's stain. The slash mark represents 25 microns.

FIGURE 4. Thick epoxy section (1 μ) through the Y-organ of an 8.5 day destalked female specimen of *Cancer irroratus*. The cells with vacuolated cytoplasm can be easily differentiated from those with non-vacuolated cytoplasm; Richardson's stain. The slash mark represents 25 microns.

TABLE I

Mean number of nuclei and reduced silver grains counted in control and bilaterally destalked animals.

Group	Number of glands	Time after destalking* (days)	Mean number of nuclei counted ± standard error**	Mean number of reduced silver grains counted ±standard error**
1°	19	0	9.07 ± 0.48	3.21 ± 0.69
2	16	2.5	7.44 ± 0.37	6.38 ± 1.21
3	17	3.5	5.05 ± 0.32	12.14 ± 1.04
4	17	5.5	5.46 ± 0.25	0.18 ± 0.56
5	18	8.5	6.07 ± 0.29	2.74 ± 0.53
6	16	11.5	6.19 ± 0.29	4.52 ± 1.38

* Time of sacrifice, 24 hrs after injection.
** Per 500 sq. microns of gland.
° Control animals, not destalked.

Sparsely scattered irregularly shaped granular lymphocytes are evident within the blood sinuses that separate the cords of Y-organ cells (Figs. 2 and 3). These blood cells are approximately 15 to 30 microns in diameter. The cytoplasm in many of these lymphocytes are densely filled with granules as to obscure the nucleus; whereas in others few granules are present. The granules are osmophilic and are quite variable in size as is the case of the granules that are evident in the Y-organ cells. Arthropod lymphocytes are known to be phagocytic (Lochhead and Lochhead, 1941); however, there is no evidence of phagocytosis or any other association between the Y-organ cells and the lymphocytes.

Cytological effects of eyestalk removal on the Y-organ

The Y-organ cells of bilaterally destalked animals show evidence of hypertrophy, the nuclei appearing larger and more ovoid in shape (Figs. 3 and 4). Because of the increase in cytoplasmic volume regions of degeneration and cytoplasmic vacuolization are more evident. Osmophilic granules appear within the cells; the numbers of such cytoplasmic granules is quite variable from cell to cell. Table I gives the mean number of nuclei counted for all glands including those from animals not bilaterally destalked. The Y-organs from the control group of animals, not bilaterally destalked, show a significantly greater number of nuclei per area counted, at $P < 0.05$, than the Y-organs from animals that have been bilaterally destalked. Tissue sections of Y-organs from crabs that have been destalked for 2–3 days (Group 2) demonstrate a significantly higher number of nuclei than those destalked for 3–4 days (Group 3) and 5–6 days (Group 4), but do not differ significantly from the last two experimental groups of glands. A smaller number of nuclei present in a unit area of gland can be an indication of greater cytoplasmic volume or cell hypertrophy. Therefore, it can be assumed that cellular hypertrophy increases steadily up to four to five days following bilateral destalking and then reaches a plateau not to return to the original condition even after 11 to 12 days after eyestalk removal.

The uptake of ^{3}H-uridine by Y-organ cells following bilateral eyestalk removal

Table I also illustrates the result of reduced silver grain counts made over randomly selected areas of Y-organs in each group of destalked and control animals. Y-organs from animals that were destalked for 3 to 4 days (Group 3) show the highest number of reduced silver grains counted; and the count in these glands is significanlty higher, at $P < 0.05$, than in all other glands counted. Y-organs from animals destalked for 2–3 days (Group 2) differ significantly only from those in Groups 3 and 4, but are significantly higher in the number of silver grains counted than the control glands (Group 1) and from those glands taken from animals in Groups 5 and 6. Glands from animals destalked for 11–12 days (Group 6) differ significantly from those in Group 4 but not from those in Group 5. There appears to be a gradual increase in the number of reduced silver grains counted up to 3 to 4 days following destalking. The level of ^{3}H-uridine incorporated after 5 to 6 days drops below the level incorporated in the control glands. By day 8–9 (Group 5) the level of uptake measured by silver grain counts approaches that in the control glands, and remains at that level even up to 11–12 days after eyestalk removal.

Discussion

Although cellular hypertrophy in the Y-organ has been observed in the Y-organ following bilateral eyestalk removal (Matsumoto, 1962; Hinsch, 1973), such hypertrophy in *Cancer irroratus* reaches its maximum level within the first four days after eyestalk removal. Following this increase in cell size, the Y-organ cells gradually decrease in size, reaching a level that remains slightly above that of the controls even after 11 to 12 days postdestalking. Paralleling the hypertrophy of the cells, there is also demonstrated an increase in the rate of RNA synthesis. This is demonstrated by the fourfold increase in the uptake of ^{3}H-uridine into the RNA of the Y-organ cells during the first four days postdestalking. Although the nature of the RNA is unknown from these experiments, it is apparent that some inhibition on RNA synthesis has been lifted by the removal of both eyestalks. The decrease in the uptake of the label five to six days postdestalking is not completely understood. It may be an indication of a certain secretory phase in the cycle of the gland. However, it is not a function of the type nor the condition of the labeled precursor that was injected into the animals; for the animals that comprised this experimental group (Group 4) received injections of labeled uridine from the same sample as those in the preceding group (Group 3). The Y-organ may be adapting to the absence of the inhibition from the eyestalk complex since the rate of RNA synthesis approximately has returned to the level of uptake demonstrated by the control animals after nine to twelve days postdestalking.

Madhyastha and Rangneker (1972) have described two cell types in the Y-organ of the crab, *Varuna litterata*. The most abundant cell type is a small epithelial cell consistent with that of *Cancer irroratus*. However, the second cell type is described as containing basophilic cytoplasmic granules and is much rarer in occurrence. It appears that this second cell type may be representative of granular lymphocytes which, in *Cancer irroratus*, can be found within the hemocoelic sinuses of the Y-organ. When the cellular cords are very compact, the

lymphocytes become compressed against them and appear to contribute to the histology of the Y-organ.

The areas of cytoplasmic vacuolization and cell degeneration in the Y-organ of *Cancer irroratus* may be evidence of its mode of secretion. Charniaux-Cotton, Zerbib and Meusy (1966) have reported such degenerate areas in the androgenic glands of crustaceans and state that they are evidences of holocrine secretion. Such described areas in the Y-organ of *Cancer irroratus* may also give evidence for holocrine activity. However, no mitotic figures were evident within the glands of *C. irroratus;* but Hoffman (unpublished observations) has noted mitotic activity within the Y-organ cells of destalked specimens of *Cancer productus.* These mitotic figures appear to be in proximity to cells with cytoplasmic vacuolization. Bressac (1973) has observed numerous lysosomal-type inclusion bodies in the Y-organ cells of *Pachygrapsus marmoratus* in electron micrograph studies. The Y-organ cells of *Cancer irroratus* of both control and destalked animals show a variability in cytoplasmic granulation. Although no quantitative measurements were taken of these granules, they appear to be more numerous in cells that contain vacuolated cytoplasm. Novikoff (1959, 1960) has noted large lysosmes or cytolysosomes in dying liver cells and the dying cells of atretic ovarian follicles. Also Scharrer (1966) has reported large irregular membrane bound bodies up to 4 microns in diameter within the degenerating prothoracic gland cells of *Leucophaea* and *Blaberus* a few days after the final molt. Scharrer believes that these bodies represent autophagic vacuoles. However, Fawcett, Long and Jones (1969) have also described cytoplasmic osmophilic granules in steroid secreting cells and indicate that these granules may be lipid droplets. Similar type granules in the Y-organ cell may give credence to the steroidogenic nature of the gland. Recently Bollenbacher and O'Connor (1973) have isolated α-ecdysone from the Y-organs of *Pachygrapsus crassipes* cultured *in vitro.* Yet, before any more definite correlations can be made concerning the precise relationship between the Y-organ and the method of synthesis and release of the crustacean molting hormone, more histochemical and biochemical data are needed, especially from a wide variety of crustaceans.

We wish to thank Ruth McDiffett and Frederick Swarts for their assistance with the statistical analysis.

Summary

1. Bilateral destalking of *Cancer irroratus* increases the rate of RNA synthesis in the Y-organ cells during the first four days postdestalking.

2. The cytoplasmic volume of the Y-organ cells also increases during the first four to five days postdestalking and then drops to a plateau that is slightly above the volume of cells from non-destalked control animals.

3. Numerous areas of cytoplasmic vacuolization and cellular degeneration are evidenced within the control and destalked crabs. It is postulated that this may be evidence of holocrine activity.

4. The Y-organ cells of *Cancer irroratus* contain numerous osmophilic granules both in control and destalked animals.

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